Antioxidant Activity of Two Phloroglucinol Derivatives from Dryopteris crassirhizoma

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The rhizome of Dryopteris crassirhizoma Nakai exhibited significant antioxidant activity, as assessed by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity in vitro. Two phloroglucinol derivatives, flavaspidic acids PB (1) and AB (2), were isolated from the rhizome of D. crassirhizoma by a bioassay-guided fractionation. 1H-, 13C-NMR, and UV analysis were used to determine the structures. Furthermore, the two compounds were tested for their antioxidant activities, such as their DPPH radical scavenging, superoxide radical scavenging, and lipid peroxidation (LPO) inhibitory activities. Compounds 1 and 2 exhibited potent antioxidant activity against the LPO inhibitory test with IC50 values of 12.9 and 13.1 μM, respectively, compared with α-tocopherol (IC50: 15.6 μM) and butylated hydroxy anisole (BHA, IC50: 10.8 μM), while the two compounds had a moderated effect on the DPPH radical scavenging activity (IC50: 71.7, 76.3 μM) as well as superoxide radical scavenging activity (IC50: 58.6, 64.4 μM). The potent activity of the flavaspidic acids (1, 2) on inhibiting LPO might be due to possible stabilization as a result of chelating with iron.

Key words Dryopteris crassirhizoma; flavaspidic acid PB; flavaspidic acid AB; antioxidant activity

MATERIALS AND METHODS

Plant Rhizome of Dryopteris crassirhizoma was collected in Mt. Sulak, Korea in July 2002 and identified by Prof. Ki-Hwan Bae, College of Pharmacy, Chungnam National University, Korea. A voucher specimen (CNU 1011) was deposited in the herbarium of the College of Pharmacy, Chungnam National University, Korea.

Isolation of Flavaspidic Acids PB (1) and AB (2) The dried rhizome (1 kg) of D. crassirhizoma was extracted with MeOH (3 l, 48 h×2) at room temperature and the MeOH solution was then concentrated to dryness in vacuo to afford a dark brown syrupy residue (150 g). The MeOH extract was suspended in H2O (1 l) and then shaken with EtOAc (1×2). The EtOAc-soluble fraction (80 g) was subjected to column chromatography on silica gel and eluted with hexane (10% acetone) and increasing amount of acetone. Five fractions were collected. The MeOH extracts from approximately 200 Korean plants were investigated in an ongoing search for natural products with potent antioxidant activity. Among them, the MeOH extract of the rhizome of Dryopteris crassirhizoma Nakai (Aspidiaceae) had a significant antioxidant effect on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity. D. crassirhizoma is a pteridophyte and its rhizome is a well known Chinese crude drug, which has been used clinically in Korea, Japan, and China as a vermicide. In addition, phloroglucinol derivatives (albaspidin, aspidin, flavaspidic acids, and dryocrassin) and kaempferol acetyl rhamnosides (crassirhizomosides A—C and sutchuenoside flavaspidic acids, and dryocrassin) and kaempferol acetyl rhamnosides (crassirhizomosides A—C and sutchuenoside flavaspidic acids, and dryocrassin) and kaempferol acetyl rhamnosides (crassirhizomosides A—C and sutchuenoside A) have been isolated from this plant source previously.2,3 The phloroglucinol derivatives possessed antibacterial,4 anti-tumor promoting,5 nitric oxide inhibitory,6 and anti-reverse transcriptase activities.7 More recently, we reported the antibacterial activity of the phloroglucinol derivatives from this plant.8 However, the antioxidant activity of the phloroglucinol derivatives isolated from D. crassirhizoma has not been reported. The aim of this study was to evaluate the antioxidant activity of the compounds isolated from D. crassirhizoma.

DPPH Radical Scavenging Activity DPPH radical scavenging activity was measured according to the procedure described by Takao et al.9 Ten microliters of each sample, dissolved in DMSO, was prepared in a 96 well plate and then 190 μl of 200 μM ethanolic DPPH solution was added. The mixture was incubated at room temperature for 30 min and the absorbance of the reaction mixture was measured at 517 nm. DPPH radical scavenging activity (%) is expressed as follows:

\[ \text{DPPH radical scavenging activity (%)} = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100 \]

Where, Acontrol is the absorbance of the control and Asample the absorbance of the test.

Superoxide (O₂⁻) Radical Scavenging Activity Superoxide was generated by xanthine/xanthine oxidase and measured by the nitroblue tetrazolium (NBT) reduction method.10,11 Fifty microliters of 4 mM xanthine, 50 μl of 225 μM NBT, 50 μl of 50 mM phosphate buffer (pH 7.8, 1 mM EDTA) and 10 μl of the test compounds were prepared in a 96 well plate, and the absorbance of each reaction mixture was measured at 517 nm. DPPH radical scavenging activity (%) is expressed as follows:

\[ \text{Superoxide radical scavenging activity (O₂⁻)} = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100 \]

Where, Acontrol is the absorbance of the control and Asample the absorbance of the test.
monitored at 550 nm. Superoxide radical scavenging activity (%) was expressed as the degree of NBT reduction decrease of the test group versus the control group after 3 min.

superoxide radical scavenging activity (%) \[ = \left( \frac{A_{\text{control}} - A_{\text{blank}}}{A_{\text{sample}} - A_{\text{blank}}} \right) \times 100 \]

Where, \( A_{\text{control}} \) is the absorbance of the control, in which the sample was not treated, \( A_{\text{sample}} \) is the absorbance of test sample which the sample was treated, and \( A_{\text{blank}} \) is the absorbance of blank, to which the sample and the NBT solution were not added. Butylated hydroxy anisole (BHA) and \( \alpha \)-tocopherol were used as positive controls.

**Preparation of Rat Brain Homogenate** The rat brain homogenate was prepared as previously described method, with some modification. Sprague–Dawley rat brains were removed, washed with ice-cold saline, homogenized in 5 volumes of ice-cold phosphate buffer (pH 7.4). The supernatant was stored at −70 °C until required for the lipid peroxidation determination.

**Lipid Peroxidation (LPO) Inhibitory Activity** Lipid peroxidation inhibitory activity in rat brain homogenate was evaluated by the thiobarbituric acid (TBA) method with some modification. The reaction mixture was composed of 10 µl of sample (DMSO), 740 µl of 50 mM-phosphate buffer (pH 7.4), 50 µl of rat brain homogenate (10 mg protein/ml) and 200 µl of the free radical generating system: 0.1 mM FeSO\(_4\)·7H\(_2\)O and 1 mM ascorbic acid. The reaction mixture was incubated at 37 °C for 30 min and the reaction was terminated by adding 250 µl of 20% trichloroacetic acid (TCA) and 250 µl of 1% TBA (in 50 mM NaOH). After boiling at 95 °C for 5 min, the mixture was centrifuged at 10000 rpm for 10 min. The absorbance of the supernatant was measured at 532 nm. Lipid peroxidation inhibitory activity (%) is expressed as follows:

lipid peroxidation inhibitory activity (%) \[ = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}} - A_{\text{blank}}} \right) \times 100 \]

Where, \( A_{\text{control}} \) is the absorbance of the control, \( A_{\text{sample}} \) is the absorbance of the sample and \( A_{\text{blank}} \) is the absorbance of the blank, to which the sample and the free radical generating system (Fe\(^{2+}\)/ascorbate) were not added.

**RESULTS AND DISCUSSION**

The EtOAc-soluble fraction of the rhizome from *D. crassirhizoma* yielded two phloroglucinol derivatives, flavaspidic acid PB (1) and flavaspidic acid AB (2), via bioassay-guided isolation using the DPPH radical scavenging activity as a model for the antioxidant activity (Fig. 1). The structures of the isolated compounds (1, 2) were identified based on their spectroscopic properties when compared to the structures reported in the literature. The two compounds were tested for their antioxidant activities, such as the DPPH and superoxide tests for their direct free radicals scavenging activities, and the LPO inhibitory activity. Their activities were then compared with those of two food antioxidants (\( \alpha \)-tocopherol and BHA). Their antioxidant activities are shown in Table 1. Compounds 1 and 2 possessed mild inhibitory activity against the DPPH radical scavenging activity with IC\(_{50}\) values of 71.7 and 76.3 µM, respectively (Table 1). In addition, the two compounds also showed mild inhibitory effect on the superoxide radical scavenging assay with IC\(_{50}\) values of 58.6 and 64.4 µM for compounds 1 and 2. However, both compounds exhibited potent inhibitory activity against the LPO inhibitory assay with IC\(_{50}\) values of 12.9 and 13.1 µM for compounds 1 and 2. This activity was a similar to \( \alpha \)-tocopherol (IC\(_{50}\); 15.6 µM) and BHA (IC\(_{50}\); 10.8 µM), which were used as the positive controls.

This study provides evidence that the two flavaspidic acids possessed interesting antioxidant properties, which was expressed by their inhibitory effect on the Fe\(^{2+}\)-induced LPO assay. It is generally assumed that the ability of phenolic compounds such as flavonoids to chelate Fe\(^{2+}\) in the LPO system is very important for their antioxidant activity. Therefore, an attempt was made to determine the role of Fe\(^{2+}\) chelation in the antioxidant activity of the phloroglucinol derivatives in the LPO assay.

The UV spectra of the two compounds (1, 2) exhibited a diagnostic 50 nm bathochromic shift of band II due to the B-ring structure in the presence of FeCl\(_3\) (Fig. 2). This suggests that a hydroxyl group at C-4’ and a carbonyl group at C-8’ of compounds 1 and 2 formed a chelate bridge with FeCl\(_3\). Rezk et al. reported the effect of an intramolecular hydrogen bridge on LPO inhibition for 2,4,6-trihydroxyacetophenone, phloretin, and phloroglucinol. In accordance with our findings, they reported that the hydrogen bond in 2,4,6-trihydroxyacetophenone, phloretin, and phloroglucinol is important for the potent LPO inhibitory activity. However, phloroglucinol (IC\(_{50}\); 624 µM), which do not form an intramolecular hydrogen bond, had a low LPO inhibitory activity. These results show that the potent activity of flavaspidic acids (1, 2) in LPO inhibition might be due to the possible stabilization resulting from chelation with iron. The potential
of using phloroglucinols as natural antioxidants has attracted more attention due to their ubiquitous occurrence in nature.

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REFERENCES